J R C T E C H N I C A L R E P O R T S

Validation of analytical methods for the WFD “watch list” pilot exercise

Validation of LC-MS/MS and GC-MS analytical methods for carbamazepine, 10,11-dihydro-10,11-dihydroxy-carbamazepine, sulfamethoxazole, pentafluoropropionic acid and tris (1-chloro-2-propyl) phosphate determination in surface water samples

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Simona Tavazzi; Gert Suurkuusk; Bruno Paracchini; Robert Loos; Giulio Mariani; Bernd Manfred Gawlik

Abbreviations

$^{13}C_6$-SMZ	Sulfamethoxazole (Ring-$^{13}C_6$)
ANOVA	Analysis of variance
CBZ	Carbamazepine
CBZ-d_{10}	Carbamazepine-d_{10}
CBZ-DiOH	10,11-Dihydro-10,11-dihydroxy-carbamazepine
CRM	Certified reference material
GC	Gas chromatography
HLB	Hydrophilic-lipophilic-balanced
IS	Internal standard
ISO	International Organisation for Standards
LoD	Limit of detection
LoQ	Limit of quantification
MRM	Multiple reaction monitoring
MS	Mass spectrometry
$^{13}C_4$-PFBA	Perfluoro-n-[1,2,3,4-$^{13}C_4$] butanoic acid
PFPrA	Pentafluoropropionic acid
PS	Priority Substances
R^2	Regression coefficient
RSD	Relative standard deviation
SD	Standard deviation
SIM	Selected ion monitoring
SMZ	Sulfamethoxazole
SPE	Solid phase extraction
TBP-d_{27}	Tris-n-butyl-d_{27} phosphate
TCPP	Tris (1-chloro-2-propyl) phosphate
UHPLC	Ultra high pressure liquid chromatography
WAX	Weak anion exchange
WFD	Water Framework Directive

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Introduction

The Water Framework Directive (WFD) 2000/60/EC (Article 16(4)) requires that the list of Priority Substances (PS) be reviewed at least every four years. PS are defined as substances presenting a significant risk to or via the aquatic environment at EU level. In order to assess risk, both hazard and exposure need to be considered. However, exposure data (environmental concentrations) may be available only in a limited geographical area, especially if a substance is not regulated. The "watch list" mechanism has been included in the new amended Environmental Quality Standards Directive under the WFD. It will ensure the targeted collection of monitoring data for emerging pollutants for the purpose of risk assessment, to support future reviews of the priority substance list. It will contain a limited number of substances (maximum up to 25) of emerging concern which will be monitored in a limited number of representative monitoring stations across Europe, to gain high-quality information on their occurrence fit for purpose for prioritization under the WFD. The list should be dynamic, to respond to new information on the potential risks posed by emerging pollutants and avoid monitoring substances for longer than necessary. Monitoring matrices will be water, sediment, or biota.

In order to contribute to the discussion and to explore how such a watch list could be organised technically and logistically, the Expert Group on Chemical Monitoring and Emerging Pollutants (CMEP) organised during 2012 a pilot exercise using the infrastructure and support provided by the European Commission's Joint Research Centre (JRC). The scope of this pilot exercise was to assess how to provide information on the presence and concentrations of selected substances of "watch list potential" using a representative EU-wide sub-set of WFD monitoring stations (ca. 250) and relying on existing infrastructure. The question of how to quickly provide suitable analytical protocols as pre-normative input, with the aim of shortening the necessary standardisation process, was also addressed.

In the context of the pilot exercise the following method validation contributed to the execution of chemical analysis of a subset of identified pilot substances (carbamazepine (CBZ), 10,11-dihydro-10,11-dihydroxy-carbamazepine (CBZ-DiOH), sulfamethoxazole (SMZ), penta-fluoropropionic acid (PFPrA) and tris (1-chloro-2-propyl) phosphate (TCPP)) in surface water samples.

CBZ-DiOH, the hydroxylated metabolite of CBZ, has been detected in waste and surface water at a higher concentration than the parent compound (De Laurentiis et al., 2012; Fenet et al., 2012; Hummel et al., 2006; Miao and Metcalfe, 2003).

CBZ, CBZ-DiOH, SMZ, and TCPP were extracted from 1L water using solid-phase extraction (SPE) with OASIS HLB adsorbent; elution was performed with ethylacetate. In contrast, PFPrA was extracted from 100 mL water using OASIS WAX cartridges (weak anion exchanger) and elution was performed with 0.1% ammonia in methanol. Determination of CBZ, CBZ-DiOH, SMZ, and PFPrA was conducted with LC-MS/MS, and TCPP with GC-MS.

This validation report will be integrated into the full study report of the pilot exercise and used for the assessment of the feasibility and difficulties encountered.

1. Materials and methods

1.1. Chemicals

1.1.1. Standards

Carbamazepine (CAS 298-46-4), lot 100M1207V, purity (TLC) 100%, retest date October 2016, code C4024, Sigma Aldrich, MO (USA);

Carbamazepine-d_{10} 100 µg/mL in acetonitrile–d3, lot SCJK-006, purity ≥ 98%, expiry date May 2015, code DLM-2806-S, Cambridge Isotope Laboratories, Inc., Andover, MA (USA);

10,11-Dihydro-10,11-dihydroxy-carbamazepine, lot 1071-050A1, purity 99.9%, retest date May 2015, Spectra 2000 SrL, Rome (Italy);

Sulfamethoxazole (CAS 723-46-6), lot 80416, purity 99.5 ± 0.5%, Dr. Ehrenstorfer, Augsburg (Germany);

$^{13}C_6$-Sulfamethoxazole (Ring-$^{13}C_6$, 99%) 100 µg/mL in acetonitrile, lot SCJI-015, purity ≥ 98%, expiry date October 2019, code CLM-6944-S, Cambridge Isotope Laboratories, Inc., Andover, MA (USA);

Pentafluoropropionic acid (CAS 422-64-0), lot 78896PM, purity 98.9%, density 1.56 g/mL, release date March 2011, code 245917, Sigma Aldrich, MO (USA);

$^{13}C_4$-Perfluoro-n-[1,2,3,4-$^{13}C_4$] butanoic acid, 50±2.5 µg/mL, lot MPFBA0911, purity >99% ^{13}C(1,2,3,4-$^{13}C_4$), expiry date September 2014, code MPFBA, Wellington Laboratories, Guelph, Ontario, Canada;

Tri-n-butyl-d_{27} phosphate (Product No. 9491.12-100-IO), Chiron AS (Norway);

Tris(1-chloro-2-propyl)phosphate, 50 µg/mL in toluene, LOT: 209071282, AccuStandard, Inc. (USA).

1.1.2. Materials and reagents

Ethyl acetate for trace analysis (Carlo Erba Reactifs-SDS);

Methanol, code 701091.1612, (LC-MS) PAI, Panreac Quimica, Barcelona (Spain);

MilliQ water obtained from a MilliQ water system, Millipore, Bedford, MA (USA);

Hexane for analysis of dioxins, furans and PCB (Sigma-Aldrich, Germany);

Ammonium acetate 99.99+%, code 431311, Sigma Aldrich, MO (USA);

Acetonitrile, code 701881.1612, (LC-MS) PAI, Panreac Quimica, Barcelona (Spain);

Acetic acid, code 07692, TraceSelectUltra for trace analysis, Sigma Aldrich, MO (USA);

Ammonium hydroxide, 28% in water, 99.99% metals basis, code 338818, Sigma Aldrich, Germnay;

OASIS HLB cartridges 6CC (0.2 g), code WAT106202, Waters, Milford, MA, USA;

OASIS WAX cartridges 6 CC (0.15 g), code 186002493, Waters, Milford, MA USA.

1.1.3. Preparation of reagent solutions

1.1.3.1. *25 mM acetic acid (for OASIS WAX)*

- 1.4 mL of glacial acetic acid was transferred into a 1 L volumetric flask and
- diluted to the volume with water.

1.1.3.2. *25 mM ammonium acetate (for OASIS WAX)*

- 1.92 g of ammonium acetate was weighed into a 1 L volumetric flask, then
- dissolved and diluted to the volume with water.

1.1.3.3. *25 mM acetate buffer pH4 (for OASIS WAX)*

- 600 mL of 25 mM acetic acid and 300 mL of 25 mM ammonium acetate were combined into a 1 L volumetric flask, then
- mixed and degased under vacuum in ultrasonic bath for 20 sec.

1.1.3.4. *0.1% ammonia in methanol (for OASIS WAX)*

- 4.48 mL of ammonium hydroxide 28% was transferred into a 1 L volumetric flask, then
- diluted to the volume with methanol and
- mixed and degased under vacuum in ultrasonic bath for 20 sec.

1.1.3.5. *Mobile phase A: 5 mM ammonium acetate*

- 0.385 g of ammonium acetate was weighed into a 1 L volumetric flask, then
- dissolved and diluted to the volume with water.

1.1.3.6. *Mobile phase B: methanol:acetonitrile 50:50 % (v/v)*

- 500 mL of methanol and 500 mL of acetonitrile were transferred into a 1 L bottle, then
- mixed and degased under vacuum in ultrasonic bath for 20 sec.

1.1.3.7. *UHPLC autosampler weak washing solutions*

- 900 mL of water, 47.5 mL of methanol, 47.5 mL of acetonitrile and 5 mL of glacial acetic acid were transferred into a 1 L bottle, then
- mixed and degased under vacuum in ultrasonic bath for 20 sec.

1.1.3.8. *UHPLC autosampler strong washing solutions*

- 45 mL of water, 475 mL of methanol, 475 mL of acetonitrile and 5 mL of glacial acetic acid were transferred into a 1 L bottle, then
- mixed and degased under vacuum in ultrasonic bath for 20 sec.

1.1.3.9. *UHPLC seal washing solutions*

- 100 mL of methanol and 900 mL of water were transferred into a 1 L bottle, then
- mixed and degased under vacuum in ultrasonic bath for 20 sec.

1.1.3.10. *UHPLC-MS/MS reconstituting solution for LC-MS/MS analysis*

- 900 mL of mobile phase A and 100 mL of mobile phase B were transferred into a 1 L bottle and mixed.

1.1.4. Preparation of standard solutions

1.1.4.1. *CBZ stock standard solution (1000 µg/mL)*

- 10 mg of CBZ was weighed into a 10 mL volumetric flask, then
- dissolved and diluted to volume with methanol and mixed.

1.1.4.2. *CBZ-DiOH stock standard solution (1000 µg/mL)*

- 10 mg of CBZ-DIOH was weighed into a 10 mL volumetric flask, then
- dissolved and diluted to volume with methanol and mixed.

1.1.4.3. *SMZ stock standard solution (1000 µg/mL)*

- 10 mg of SMZ was weighed into a 10 mL volumetric flask, then
- dissolved and diluted to volume with methanol and mixed.

1.1.4.4. *Intermediate standard solution 1 (CBZ, CBZ-DiOH, SMZ 1 µg/mL)*

- 10 µL of CBZ, CBZ-DiOH and SMZ stock standard solutions (1000 µg/mL) were transferred into a 10 mL volumetric flask, then
- diluted to volume with methanol and mixed.

1.1.4.5. *Intermediate standard solution 2 (CBZ, CBZ-DiOH, SMZ 10 ng/mL)*

- 0.1 mL of CBZ, CBZ-DiOH and SMZ intermediate standard solution 1 (1 µg/mL) were transferred into a 10 mL volumetric flask, then
- diluted to volume with methanol and mixed.

1.1.4.6. *TCPP working standard solution (1 µg/mL)*

- 0.2 mL of TCPP standard solution (50 µg/mL) was transferred into a 10 mL volumetric flask, then
- diluted to volume with acetone:methanol 50:50 % (v/v) and mixed.

1.1.4.7. *Standard solution A (CBZ, CBZ-DiOH, SMZ: 0.2 ng/mL, TCPP 10 ng/mL)*

- 20 µL of intermediate standard solution 2 was transferred into a 1 mL dark vial, then
- 1 µL of TCPP working solution was added and after
- diluted to 1 mL with acetone:methanol 50:50 % (v/v) and mixed.

1.1.4.8. *Standard solution B (CBZ, CBZ-DiOH, SMZ, 1 ng/mL, TCPP 25 ng/mL)*

- 0.1 mL of intermediate standard solution 2 was transferred into a 2 mL dark vial, then
- 25 µL of TCPP working solution was added and after
- diluted to 1 mL with acetone:methanol 50:50 % (v/v) and mixed.

1.1.4.9. *Standard solution C (CBZ, CBZ-DiOH, SMZ, 2 ng/mL, TCPP 50 ng/mL).*

- 0.2 mL of intermediate standard solution 2 was transferred into a 2 mL dark vial, then
- 50 µL of TCPP working solution was added and
- diluted to 1 mL with acetone:methanol 50:50 % (v/v) and mixed.

1.1.4.10. *Standard solution D (CBZ, CBZ-DiOH, SMZ, 40 ng/mL, TCPP 200 ng/mL)*

- 40 µL of intermediate standard solution 1 was transferred into a 2 mL dark vial, then
- 0.2 mL of TCPP working solution was added and
- diluted to 1 mL with acetone:methanol 50:50 % (v/v) and mixed.

1.1.4.11. *Standard solution E (CBZ, CBZ-DiOH, SMZ, 100 ng/mL, TCPP 500 ng/mL)*

- 0.1 mL of intermediate standard solution 1 was transferred into a 2 mL dark vial, then
- 0.5 mL of TCPP working solution was added and
- dilute to 1 mL with acetone:methanol 50:50 % (v/v) and mixed.

1.1.4.12. *Standard solution low QC (CBZ, CBZ-DiOH, SMZ, 3 ng/mL, TCPP 30 ng/mL)*

- 0.3 mL of intermediate standard solution 2 was transferred into a 2 mL dark vial, then
- 30 µL of TCPP working solution was added and
- diluted to 1 mL with acetone:methanol 50:50 % (v/v) and mixed.

1.1.4.13. *Standard solution high QC (CBZ, CBZ-DiOH, SMZ, 90 ng/mL, TCPP 300 ng/mL)*

- 0.09 mL of intermediate standard solution 1 was transferred into a 2 mL dark vial, then
- 0.3 mL of TCPP working solution was added and
- diluted to 1 mL with acetone:methanol 50:50 % (v/v) and mixed.

1.1.4.14. *PFPrA stock standard solution (1000 µg/mL)*

- 6.4 µL of PFPrA was transferred into a 10 mL volumetric flask, then
- dissolved and diluted to volume with water and mixed.

1.1.4.15. *PFPrA intermediate standard solution 1 (10 µg/mL)*

- 10 µL of PFPrA stock standard solution was transferred into a 10 mL volumetric flask, then
- dissolved and diluted to volume with water and mixed.

1.1.4.16. *PFPrA intermediate standard solution 2 (500 ng/mL)*

- 1 mL of PFPrA stock standard solution was transferred into a 20 mL volumetric flask, then
- dissolved and diluted to volume with water and mixed.

1.1.4.17. *PFPrA intermediate standard solution 3 (100 ng/mL)*

- 1 µL of PFPrA stock standard solution was transferred into a 10 mL volumetric flask, then
- dissolved and diluted to volume with water and mixed.

1.1.4.18. *PFPrA intermediate standard solution 4 (10 ng/mL)*

- 0.1 mL of PFPrA stock standard solution was transferred into a 10 mL volumetric flask, then
- dissolved and diluted to volume with water and mixed.

1.1.4.19. *Internal standard working solution (CBZ-d_{10} and $^{13}C_6$-SMZ ,1 µg/mL)*

- 0.1 mL of CBZ-d_{10} 100 µg/mL and 0.1 mL of $^{13}C_6$-SMX 100 µg/mL were transferred into a 10 mL volumetric flask, then
- diluted to 10 mL with methanol and mixed.

1.1.4.20. *Internal standard working solution (TBP-d_{27}, 10 µg/mL)*

- 1.0 mL of TBP-d_{27} 100 µg/mL was transferred into a 10 mL volumetric flask, then
- diluted to 10 mL with hexane and mixed.

1.1.4.21. *Internal standard working solution ($^{13}C_4$-PFBA, 50 ng/mL)*

- 0.5 mL of $^{13}C_4$-PFBA 100 µg/mL was transferred into a 10 mL volumetric flask, then
- diluted to 10 mL with methanol and mixed.

1.2. Apparatus

1.2.1. Laboratory equipment

Analytical balance: Model AX204, Mettler-Toledo SpA;

Automatic pipettes: Eppendorf research (Milan, Italy);

Microsyringes: Microliter Syringes, Hamilton (Reno, CA, U.S.A.);

Autosampler vials for GC-MS: V-Vial 11 mm, Vol. 1,5 mL, 12 x 32 mm (code ARO-3741-12) with 11 mm 0.040" PTFE/Silicone Septa silver crimp cap (code ARO-5750-13), Phenomenex (United Kingdom);

Autosampler vials for LC-MS: Micro-V vials target Dp clear, 2 mL, 12x22 mm, National Scientific (Germany);

Volumetric flasks: Grade A, various sizes, Duran®;

Volumetric pipettes: Grade A, various sizes, Duran®;

Dionex Autotrace AT280 automated SPE system (Thermo Scientific, Waltham, MA, USA);

TurboVap II (Caliper Life Science, Mountain View, CA, USA);

Vortex Genius, Ika, Staufen, Germany.

1.2.2. Instrumental equipment and conditions

1.2.2.1. *UHPLC equipment and conditions*

The technical information and the working conditions of the UHPLC system are shown in the following Table 1 and the elution gradient scheme used in the Table 2 below.

Table 1 Equipment and conditions of UHPLC system

Pumps:	Binary Solvent Manager, Model UPB, Waters (Milford, MA, USA).
Autosampler:	Sample Manager, Model UPA, Waters (Milford, MA, USA).
Detector:	QTRAP 5500, Applied Biosystems MDS SCIEX, (Foster City, CA, U.S.A) equipped with Turbo V™ ion source.
Flow rate:	400 µL/min
Injection volume:	5 µL
Analytical column:	Triart C18, 1.9 µm, 50 x 2.1 mm, YMC (Dinslaken, Germany) equipped with UHPLC column saver (Fortis, Technologies)
Mobile phase:	5 mM ammonium acetate – acetonitrile -methanol (90:5:5 % (v/v/v))

Table 2 Gradient mode scheme of UHPLC system

Time (min)	A	B	Flow (mL/min)
0	90	10	0.4
1	90	10	0.4
9	5	95	0.4
9.1	5	95	0.4
9.2	90	10	0.4
12	90	10	0.4

Under these conditions the retention times of CBZ, CBZ-DiOH, SMZ and PFPrA were about 6.0, 4.6, 2.9 and 1.3 min, respectively. The run time was about 12.5 min.

1.2.2.2. *QTRAP 5500 operative condition*

An AB Sciex QTRAP 5500 mass spectrometer equipped with Turbo V™ ion source was used. The instrument was previously tuned and calibrated in electrospray mode using PPG's. Prior to analysis, all the specific parameters were optimised infusing a 1 µg/mL standard solution of analytes and ISs. The instrument was run (for quantification) in the MRM MS-MS mode.

The eluent from the column was introduced directly into the ion source. The rapid desolvation and vaporization of the droplets minimizes thermal decomposition and preserved their molecular identity.

The data were collected using the software program Analyst 1.5.1.

All calculations were based on chromatographic peak area ratios for the MRM precursor-product ion transitions for analytes versus ISs.

The general operating conditions of QTRAP are shown in the following Table 3

Table 3 Operating conditions of QTRAP 5500

Scan Type:	Scheduled MRM
Polarity:	Positive for CBZ, CBZ-DiOH and SMZ
Ion Source:	Turbo Spray
Resolution Q1:	Unit
Resolution Q3:	Unit
MR Pause:	5.0000 msec

Analyte	Time(min)	DP	CE
CBZ (237>194)	6	250	28
CBZ (237>165)	6	250	60
CBZ-DiOH (271>180)	4.6	80	47
CBZ-DiOH (271>180)	4.6	80	19
SMZ (254>156)	2.9	150	22
SMZ (254>156)	2.9	150	38

CUR:	25.00
CAD:	Medium
TEM:	550.00
IS:	+4500.00
EP	+10.00
CXP	+13.00
GS1	55
GS2	45

Scan Type:	Scheduled MRM
Polarity:	Negative for PFPrA
Ion Source:	Turbo Spray
Resolution Q1:	Unit
Resolution Q3:	Unit
MR Pause:	5.0000 msec

Analyte	Time(min)	DP	CE
PFPrA (163>119)	1.3	-113	-20
PFPrA (163>69)	1.3	-113	-45

CUR:	25.00
CAD:	Medium
TEM:	550.00
IS:	-4500.00
EP	-10.00
CXP	-11.00
GS1	55
GS2	45

1.2.2.3. *GC-MS equipment and conditions*

The technical information and the working conditions of the GC-MS system are shown in the following Table 4.

Table 4 Equipment and conditions of GC-MS system

GC	Agilent 6890 N			
Column:	SGE-BPX-50			
Nominal length:	60 m			
Nominal Diameter:	250 µm			
Nominal film thick-ness:	0.25 µm			
Gas Type	Helium			
Mode:	constant flow			
Initial flow:	1 mL/min			
Oven:				
Initial Temperature:	80℃			
Initial Time:	1 min			
Ramps:	#	Rate	Final Temp	Final Time
	1	30℃/min	180℃	0 min
	2	10℃/min	300℃	15 min
Run Time	31.33 min			
Front Inlet (CIS4)				
Mode	Splitless			
Initial Temperature	250℃			
Pressure	180 kPa			
Purge Flow	100 mL/min			
Purge Time	1 min			
Total Flow	104.1 mL/min			
Gerstel CIS 4				
Initial Temperature	80℃			
Equilibration Time	0.05 min			
Initial Time	0.10 min			
Rate	10 ℃/sec			
Final Temp	280 ℃			
Hold Time	10 min			
MS	Agilent 5973 Mass Selective Detector			
Mode	EI			
MS Quad	150 ℃			
MS Source	230 ℃			

2. Preparation of calibration standards and QC samples

2.1. Calibration standards and QC samples for CBZ, CBZ-DiOH, SMZ and TCPP

- 1 L glass bottle was filled with 1L MilliQ water and
- 1 mL of working standard solutions were added according to the following scheme shown in Table 5:

Table 5 Scheme of calibration standards and QC samples for CBZ, CBZ-DiOH, SMZ and TCPP

Working solution	CBZ[a], CBZ-DiOH[a], SMZ[a], TCPP[b] concentration ng/mL	CBZ, CBZ-DiOH, SMZ, concentration in water ng/L	Sample type
A	0.2[a], 10[b]	0.2[a], 10[b]	Calibration samples
B	1[a], 25[b]	1[a], 25[b]	„
C	2[a], 50[b]	2[a], 50[b]	„
D	40[a], 100[b]	40[a], 100[b]	„
E	100[a], 500[b]	100[a], 500[b]	„
Low QC	3[a], 30[b]	3[a], 30[b]	QC samples
High QC	90[a], 300[b]	90[a], 300[b]	„

2.2. Preparation of water samples for CBZ, CBZ-DiOH, SMZ and TCPP

- 10 µL of IS working solution of CBZ-d10, $^{13}C_6$-SMZ and TBP_{d27} were added to 1 L water standard and QC samples.
- Samples were shaken.
- SPE OASIS HLB cartridges were conditioned with 10 mL of ethyl acetate.
- SPE cartridges were conditioned with 10 mL of methanol.
- SPE cartridges were conditioned with 10 mL of water.
- Water samples were loaded at flow 10 mL/min.
- Sorbent was dried under nitrogen flow for 30 min.
- Samples were eluted with 10 mL ethyl acetate at flow 5 mL/min.

Half of received extract (i.e. about 5 mL) was evaporated to dryness and reconstituted in 0.2 mL of reconstituting solution for LC-MS/MS analysis. The remaining aliquot (i.e. about 5 mL) was evaporated to 50-100 µL under nitrogen flow for GC-MS determination.

2.3. Preparation of calibration standards and QC samples for PFPrA

- 1 L glass bottle was filled with 0.1L MilliQ water.
- Working standard solutions were added according to the following scheme shown in Table 6:

Table 6 Scheme of calibration standards and QC samples for PFPrA

Working solution	PFPrA concentration ng/mL	Added volume (mL)	PFPrA, concentration in water ng/L	Sample type
Intermediate solution 2	10	0.01	1	Calibration samples
Intermediate solution 2	10	0.05	5	„
Intermediate solution 3	50	0.02	10	„
Intermediate solution 3	50	0.04	20	„
Intermediate solution 4	500	0.02	100	„
Intermediate solution 2	500	0.04	200	
Low QC	10	0.3	3	QC samples
High QC	500	0.3	150	„

2.4. Preparation of water samples for PFPrA

The analytical method for PFPrA was adapted from the methods published by Li et al. (2010) and Taniyasu et al. (2005; 2008), and ISO method 25101 (2009).

- 10 µL of IS working solution of $^{13}C_4$-PFBA was added to 0.1 L water standard and QC samples.
- Samples were shaken.
- SPE OASIS WAX cartridges were conditioned with 4 mL of 0.1% ammonia in methanol.
- SPE cartridges were conditioned with 4 mL of methanol.
- SPE cartridges were conditioned with 4 mL of water.
- Water samples were loaded at flow 10 mL/min
- Cartridges were washed with 4 mL 25 mM acetate buffer pH 4.
- Sorbent was dried under nitrogen for 20 min.
- Samples were eluted with 4 mL of 0.1% ammonia in methanol.

The extract was evaporated to dryness and reconstituted in 200 µL of reconstituting solution for LC-MS/MS analysis.

3. Validation procedure and results

Validation was carried out according to the ISO 17025 standard. During the validation process following parameters were evaluated: selectivity, method linearity and working range, recovery, limits of detection and quantification, trueness, repeatability, reproducibility and intermediate precision. Finally the relative combined standard uncertainty was estimated.

3.1. Selectivity

3.1.1. CBZ, CBZ-DiOH, SMZ, PFPrA and corresponding standards

For the identification of CBZ, CBZ-DiOH, SMZ and PFPrA two MRM transitions between the precursor ions and two most abundant fragment ions were monitored. The first one was used for quantification purposes, whereas the second one was to confirm the presence of the target compounds in the sample. The quantified analyte was identified through retention time comparison of the corresponding standard and the isotopic ratio between two ions recorded (±30%), in comparison to the standard.

The selected mass transitions used for quantification were 237/194 for CBZ, 271/180 for CBZ-DiOH, 254/156 for SMZ, 247/204 for CBZ-d_{10}, 260/98 for $^{13}C_6$-SMZ, 163/119 for PFPrA and 217/172 for $^{13}C_4$-PFBA.

3.1.2. TCPP and corresponding standard

TCPP (sum of three isomers) was identified in SIM (Selected Ion Monitoring) mode, recording the following ion traces: 277 and 279 amu (two isotopic ions of the cluster Cl). The quantified analyte was identified through retention time comparison of the corresponding standard and the isotopic ratio between two ions recorded (±20%).

For TBP-d_{27} identification following ion traces were monitored: 103 amu and 167 amu.

3.2. Limit of Detection (LoD) and Limit of Quantification (LoQ)

Limits of detection and quantification were estimated by analysing blank samples. From the data achieved from these experiments (n = 5-7) the mean value of the blank samples (b) and standard deviation (SD) were calculated. LoD and LoQ were estimated using the following formulas, recommended by Eurachem Guide (Eurachem Group, 1998).

LOD=b+3SD;

LOQ=b+10SD.

The results of the LoD and LoQ estimation for every analyte are shown in the Table 7.

Table 7 LoD and LoQ values for analytes

Analyte	Nr of blanks analyzed	LoD (ng/L)	LoQ (ng/L)
CBZ	5	0.03	0.07
CBZ-DiOH	5	0.10	0.26
SMZ	5	0.05	0.13
TCPP	7	2.00	4.00
PFPrA	5	0.59	1

3.3. Linearity study

3.3.1. Linearity study for CBZ, CBZ-DiOH and SMZ

For CBZ, CBZ-DiOH and SMZ the linearity was studied in the concentration range 0.2-100 ng/L.

In order to verify the linearity of the calibration curve, a blank sample spiked only with labelled internal standard and five spiked MilliQ water samples (i.e.: 0, 0.2, 1, 2, 40, 100 ng/L) were extracted and analyzed in three replicates on five different days. The calibration curves are reported in Annex 1: Figure 1 for CBZ, Figure 3 for CBZ-DiOH and Figure 5 for SMZ.

As reported in Table 8, the mean coefficient of determination (R^2) values, calculated over five calibration curves, were ≥0.99 for every analyte, with RSD% of 0.4, 0.6 and 0.9 for CBZ, CBZ-DiOH and SMZ, respectively.

Table 8 Coefficient of determination (R^2) values for CBZ, CBZ-DiOH and SMZ calibration curves on different days

Validation day	CBZ	CBZ-DiOH	SMZ
1	1.0000	0.9962	0.9988
2	0.9993	0.9991	0.9986
3	0.9988	0.9893	0.9977
4	0.9993	0.9868	0.9991
5	0.9900	0.9860	0.9784
Average	0.9975	0.9915	0.9945
RSD%	0.4	0.6	0.9

The study of the distribution of residuals revealed randomly dispersed shapes around the horizontal axis, proving the pertinence of the linear regression model for interpreting the data.

The received residual plots are reported in Annex 1 (Figure 2 (CBZ), Figure 4 (CBZ-DiOH) and Figure 6 (SMZ)).

3.3.1.1. *Extension of the calibration for CBZ, CBZ-DiOH and SMZ analysis*

The upper limit of the calibration curve was initially set at 100 ng/L. However, in order to be able to quantify real water samples with analytes concentration above this reported upper limit of the calibration curve (100 ng/L), an extended calibration curve (up to 2000 ng/L) was evaluated. A blank sample spiked only with labelled internal standard and six spiked MilliQ water samples (i.e.: 0, 0.2, 2, 40, 100, 1000, 2000 ng/L) were extracted and analysed in three replicates on a single day basis. Even in the wider concentration range, the coefficient of determination (R^2) resulted to be >0.99 and the residual plot showed a random distribution against x axis. The calibration curves of the extended linearity study are shown in Annex 1, Figure 11.

3.3.2. Linearity study for TCPP

For TCPP the linearity was studied in the concentration range 0-500 ng/L.

In order to verify the linearity of the calibration curve, a blank sample spiked only with labelled internal standard and five spiked MilliQ water samples (i.e.: 0, 10, 25, 50, 200 and 500 ng/L) were extracted and analysed in three replicates on eight different days. The received calibration curves are shown in Annex 1, Figure 7.

The linearity of the calibration plots was estimated by calculating the regression coefficient R^2 and by checking the shape of distribution of residuals. The calculated R^2 values were ≥0.99 for all

calibration curves (see Table 9) and the residuals were randomly dispersed around the horizontal axis (see the residual plots for TCPP in the Annex 1, Figure 8).

Table 9 Coefficient of determination (R^2) values for TCPP calibration curves on different days

Validation day	TCPP
1	0.9947
2	0.9999
3	1.0000
4	0.9989
5	0.9990
6	0.9996
7	0.9999
8	0.9992
Average	0.9989
RSD %	0.18%

The linear regression model proved to be appropriate for interpreting the data.

3.3.2.1. *Extension of the calibration for TCPP analysis*

According to the information received from scientific literature (Andresen, et al., 2004; Bollmann, et al., 2012; Regnery, et al., 2010) the linearity of the method was first tested in the concentration range from 0 ng/L to 500 ng/L as described in the paragraph 3.3.2. After finalising the measurements of the real samples it was realised that around 15% of the results were exceeding the concentration of the maximum calibration point. In order to check the linearity of the method in higher concentration level, additional experiments were carried out and the linearity study was extended to 3000 ng/L by analysing 6 calibration solutions with concentrations 10, 50, 200, 750, 1500 and 3000 ng/L. The calibration solutions were prepared by spiking 1L of MilliQ water with native TCPP and extracted by SPE like real samples.

Also in this case the established R^2 value was >0.99. It can be stated, that inside the tested concentration range the method is linear. The calibration curve of the extended linearity study is shown in the in Annex 1, Figure 11.

3.3.3. Linearity study for PFPrA

For PFPrA the linearity was studied in the concentration range 1-200 ng/L.

In order to verify the linearity of the calibration curve, a blank sample spiked only with labelled internal standard and six spiked MilliQ water samples (i.e.: 0, 1, 5, 10, 20, 100, 200 ng/L) were extracted and analysed in three replicates on five different days.

The received calibration curves are reported in Annex 1, Figure 9.

The mean R^2 values (calculated over five calibration curves) were ≥0.99. Results are reported in Table 10.

Table 10 Coefficient of determination (R^2) values for PFPrA calibration curves on different days

Validation day	PFPrA
1	0.9990
2	0.9990
3	0.9970
4	0.9970
5	0.9980
Average R^2	0.9980
RSD%	0.1

The study of the distribution of residuals revealed randomly dispersed shapes around the horizontal axis, proving the pertinence of the linear regression model for interpreting the data.

The received residual plots are reported in Annex 1, Figure 10.

3.4. Working Range

The working range is defined as the range of concentrations where the chosen calibration curve is valid. The working range of these methods was therefore defined by the limits of quantification and highest points in the respective calibration curve. Table 11 summarizes the working ranges established for the developed procedures.

Table 11 Working ranges of the analytes

Analyte	Working range (ng/L)
CBZ	0.07-2000
CBZ-DiOH	0.26-2000
SMZ	0.13-2000
TCPP	4.0-3000
PFPrA	1.0-200

3.5. Trueness

The significance test (e.g. t-test) was used to decide whether the difference between the mean values of spiked water quality control samples (evaluated on n replicates) and their nominal concentration was significant, using the following formula:

$$\mathbf{t} = \frac{(\mathbf{x} - \mu) \times \sqrt{\mathbf{n}}}{\boldsymbol{s}}$$

where *(x)* is the mean value of *(n)* samples with standard deviation *(s)* and *(μ)* is the nominal concentration. The confidence level for critical t-values were chosen to be 0,05 (95%).

3.5.1. CBZ, CBZ-DiOH and SMZ

Fifteen quality control samples at low and high concentration levels (i.e.: spiked at about 3 and 90 ng/L) were extracted and analysed and the back calculated concentrations (using internal surrogate standards) evaluated for demonstrating the truthfulness of the null hypothesis (H_0: the analytical method is not subject to systematic error).

3.5.2. TCPP

Eight spiked water samples with concentration of 30 ng/L and eight spiked water samples with concentration of 300 ng/L were analysed and the back calculated concentrations evaluated for demonstrating the truthfulness of the null hypothesis (H_0: the analytical method is not subject to systematic error).

3.5.3. PFPrA

Fifteen quality control samples at low and high concentration level (i.e.: spiked at about 3 and 150 ng/L) were extracted and analysed and the back calculated concentrations evaluated for demonstrating the truthfulness of the null hypothesis (H_0: the analytical method is not subject to systematic error).

As reported in Table 12, the calculated t-values resulted to be lower than the critical values for all the analytes at all the studied concentration levels, demonstrating the absence of evidence of systematic errors in analyte quantification.

Table 12 Results of the trueness study in the different concentration levels

Analyte	Mean value (x) ng/L	Nr of samples (n)	Nr of degrees of freedom	Theoretical value (μ) ng/L	SD of samples (s) ng/L	Calculated t-value	Critical t_4 P=0.05	Decision
CBZ	3.2	15	14	3.1	0.43	1.09	2.14	OK
	91.3	15	14	93.6	10.4	-0.87	2.14	OK
CBZ-DiOH	2.9	15	14	2.9	0.378	0.10	2.14	OK
	91.8	15	14	88.2	9.694	1.44	2.14	OK
SMZ	3.5	15	14	3.4	0.378	0.82	2.14	OK
	104.5	15	14	101.7	9.694	1.12	2.14	OK
TCPP	34.1	8	7	30	5.33	2.19	2.36	OK
	324	7	6	300	37.37	1.71	2.36	OK
PFPrA	3.1	15	14	3	0.25	0.96	2.14	OK
	150.1	15	14	150	11.98	0.02	2.14	OK

3.6. Recovery

Recovery was evaluated by extracting and analysing in triplicate MilliQ water samples (1 L volume for CBZ, CBZ-DiOH, SMZ, TCPP and 0.1 L volume for PFPrA) spiked, before extraction, with native analytes only. Internal standards were then added to the extracts at the end of sample preparation with the aim to allow the estimation of analytes loss during processing.

The recovery was evaluated comparing the ratios analyte/IS in spiked samples to the same ratios obtained by analysing a standard solution containing native compounds and labelled ones at the same concentration levels, not subject to any handling.

The spiking levels were 3 and 90 ng/L for CBZ, CBZ-DiOH, and SMZ, 30 and 300 ng/L for TCPP and 30 and 150 ng/L for PFPrA.

The results of recovery experiments are reported in Table 13.

Table 13 Recovery of analytes at different concentration levels

Analyte	Spike level	Mean Recovery (%) (n=9)	SD (ng/L)	RSD%
CBZ	Low	84	4.0	4.7
	High	88	2.0	2.3
CBZ-DiOH	Low	74	5.7	7.6
	High	85	5.1	6
SMZ	Low	74	12.1	16.3
	High	97	9.2	9.5
TCPP	Low	126	2.3	1.8
	High	106	2.9	2.7
PFPrA	Low	120	15.3	12.7
	High	95	7.2	7.5

3.7. Repeatability, intermediate precision, and day to day variation

For repeatability, intermediate precision and day to day variation estimation, quality control samples at two concentration levels (i.e. spiked MilliQ water samples) were tested on five different days. For each sample three replicate injections were made. Using one-way ANOVA the results were obtained as shown in Table 14.

Table 14 Repeatability, day-to-day and intermediate precision variation in two different concentration levels

Analyte	Lower concentration level			Higher concentration level		
	Repeatability (%)	Day-to-day variation (%)	Intermediate precision (%)	Repeatability (%)	Day-to-day variation (%)	Intermediate precision (%)
CBZ	3.7	13.6	14.1	9.4	7.0	11.7
CBZ-DiOH	3.2	13.4	13.8	2.7	1.0	2.9
SMZ	11.2	9.0	14.4	7.6	8.1	11.2
TCPP	2.5	6.5	6.9	1.1	5.9	6.0
PFPrA	8.1	6.5	2.4	7.9	11.4	13.8

3.8. Sample storage stability study

Stability of analytes in water samples during storage was studied by analysing low and high QC samples (spiked MilliQ water), prepared on August 03, 2012 and stored at identical temperature and lighting conditions (i.e.: +5°C, darkness) as the real water samples.

Stability samples for CBZ, CBZ-DIOH and SMZ were extracted and analysed on day 0, 38, 83 and 96 after spiking.Stability samples for TCPP were extracted and analysed after 40, 84 and 97 days.

Concentrations in stability samples fall within ± 2 times the standard deviation of the concentrations of quality control samples used for repeatability evaluation.

The stability study covers the time elapsed from the collection of the first sample to the end of the analytical work of the project.

It was concluded that all target analytes are stable under the storage conditions.

A graphical representation of stability data is reported in Annex 1, Figure 12 (for CBZ, CBZ-DiOH, SMZ) and Figure 13 (for TCPP).

3.9. Uncertainty estimation

The estimation of measurement uncertainty was carried out following an alternative ("top-down") approach based on in-house validation data. The data derived from the validation of the method includes the sample preparation, standard dilution, and chromatographic and MS detection variability, measured as RSD (relative standard deviation). According to this approach the two main sources of uncertainty are method trueness and precision. The uncertainty of prepared standard stock solution is taken into account as other source of uncertainty. The expanded uncertainty was calculated using the following formula:

$$\mathbf{U_c = \pm k\sqrt{(u_{Prec})^2 + (u_{Tness})^2 + (u_{Std})^2}}$$, where:

$\mathbf{u_{Prec}}$ is standard relative uncertainty associated to the precision and is derived from the relative intermediate precision of measurements of QC samples.

$\mathbf{u_{Tness}}$ is standard relative uncertainty associated to the trueness and has been calculated from relative standard deviation of the mean of QC samples used for the trueness study and relative bias as follows:

$$\mathbf{u_{Tness} = \sqrt{\left(\frac{SD}{C\sqrt{n}}\right)^2 + \left(\frac{C - C_{Theor}}{C}\right)^2}}$$, where:

SD is the standard deviation of the results of QC samples analyses,

C is the average result of the QC samples analyses,

n is the number of QC samples that have been analysed and

$\mathbf{C_{Theor}}$ is the theoretical concentration of the QC sample.

$\mathbf{u_{Std}}$ is standard relative uncertainty associated to the used certified materials calculated as follows.

In case of TCPP analysis:

$$\mathbf{u_{Std} = \sqrt{(u_{TCPP})^2 + (u_{IS})^2 + (u_{Flask})^2 + 2\left(u_{Syringe}\right)^2}}$$, where:

$\mathbf{u_{TCPP}}$ is uncertainty of the certified standard solution of TCPP,

$\mathbf{u_{IS}}$ is the uncertainty of the certified standard solution of TBP-d_{27},

$\mathbf{u_{Flask}}$ is uncertainty related to the volumetric flask, a rectangular distribution is assumed to obtain a standard uncertainty of flask and

$\mathbf{u_{Syringe}}$ is the uncertainty related to the withdrawn of standard solution, from syringe accuracy a rectangular distribution is assumed to obtain a standard uncertainty.

In case of CBZ, CBZ-DiOH and SMZ analysis:

$$\mathbf{u_{Std} = \sqrt{(u_{balance})^2 + (u_{Flask})^2}}$$, where:

u_{Flask} is uncertainty related to the volumetric flask, a rectangular distribution is assumed to obtain a standard uncertainty of flask and

$u_{balance}$ is the uncertainty related to the weighing of crystalline powder, from balance accuracy a rectangular distribution is assumed to obtain a standard uncertainty.

k is correlation coefficient (k=2).

As the precision and trueness of the method were estimated in two different concentration levels, the uncertainty can also be estimated separately for the low and high concentration levels.

The detailed uncertainty budgets and results of the uncertainty estimations are reported in Table 15 (for CBZ, CBZ-DiOH and SMZ) in Table 16 (for PFPrA) and Table 17 (for TCPP).

Table 15 Uncertainty budget and estimated uncertainty for CBZ, CBZ-DiOH and SMZ analysis

Sources of uncertainty			**CBZ**		**CBZ-DiOH**		**SMZ**	
	Abbreviation	Unit	Low conc.	High conc.	Low conc.	High conc.	Low conc.	High conc.
Intermediate precision		%	14.1	11.7	13.8	10.4	14.4	16.1
Standard relative uncertainty associated to the precision	u_{Pres}		0.141	0.117	0.138	0.104	0.144	0.161
Average concentration of the QC samples	C	ng/L	3.24	91.26	2.95	100.35	3.47	110.35
Standard deviation of the QC samples	SD	ng/L	0.45	8.07	0.4	10.1	0.38	14.12
Number of the QC samples	n		15	15	15	15	15	15
Theoretical concentration of the QC sample	C_{Theor}	ng/L	3.12	93.6	2.94	98	3.39	113
Standard relative uncertainty associated to the trueness	u_{Tness}		0.07	0.042	0.06	0.08	0.054	0.044
Standard relative uncertainty associated to crystalline standard weighing	$u_{Balance}$		0.07	0.07	0.07	0.07	0.07	0.07
Accuracy of the volumetric flask		mL	0.04	0.04	0.04	0.04	0.04	0.04
Standard relative uncertainty associated to the accuracy of the volumetric flask	u_{Flask}		0.0231	0.0231	0.231	0.231	0.231	0.231

Standard relative uncertainty associated to the preparation of the standard stock solution	u_{Std}		0.074	0.074	0.074	0.074	0.074	0.074
Correlation coefficient	k		2	2	2	2	2	2
Expanded combined relative uncertainty	**U_c**	**%**	**35**	**39**	**34**	**31**	**34**	**36**

Table 16 Uncertainty budget and estimated uncertainty for PFPrA analysis

Sources of uncertainty	Abbreviations	Unit	Low concentration level	High concentration level
Intermediate precision		%	10.4	13.8
Standard relative uncertainty associated to the precision	u_{Pres}		0.104	0.138
Average concentration of the QC samples	C	ng/L	3.06	150.07
Standard deviation of the QC samples	SD	ng/L	0.305	14.931
Number of the QC samples	n		15	15
Theoretical concentration of the QC sample	C_{Theor}		3	150
Standard relative uncertainty associated to the trueness	u_{Tness}		0.031	0.034
Standard relative uncertainty associated to standard weighing	$u_{Balance}$		0.07	0.07
Accuracy of the volumetric flask		mL	0.04	0.04
Standard relative uncertainty associated to the accuracy of the volumetric flask	u_{Flask}		0.0231	0.0231
Standard relative uncertainty associated to the preparation of the standard stock solution	u_{Std}		0.074	0.074
Correlation coefficient	k		2	2
Expanded combined relative uncertainty of the method	**U_c**	**%**	**26**	**36**

Table 17 Uncertainty budget and estimated uncertainty for TCPP analysis

Sources of uncertainty	Abbrevi-ations	Unit	Low conc.	High conc.
Intermediate precision		%	6.9	6.0
Standard relative uncertainty associated to the precision	u_{Pres}		0.069	0.060
Average concentration of the QC samples	C	ng/L	34.04	324.16
Standard deviation of the QC samples	SD	ng/L	5.39	37.37
Number of the QC samples	n		8	7
Theoretical concentration of the QC sample	C_{Theor}	ng/L	30	300
Standard relative uncertainty associated to the trueness	u_{Tness}		0.1312	0.0863
Guaranteed accuracy of the TCPP certified standard solution		%	0.5	0.5
Standard relative uncertainty associated to the accuracy of the standard of TCPP	u_{TCPP}		0.005	0.005
Uncertainty in the preparation of the TBP-d27 certified standard solution	u_{IS}		0.05	0.05
Accuracy of the volumetric flask		mL	0.04	0.04
Standard relative uncertainty associated to the accuracy of the volumetric flask	u_{Flask}		0.0231	0.0231
Accuracy of the syringe		%	1	1
Standard relative uncertainty associated to the accuracy of the syringe	$u_{Syringe}$		0.0058	0.0058
Standard relative uncertainty associated to the preparation of the standard stock solution	u_{Std}		0.0565	0.0565
Correlation coefficient	k		2	2
Expanded combined relative uncertainty of the method	**U_c**	**%**	**32**	**24**

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ANNEX 1

Figure 1 Calibration curves of CBZ

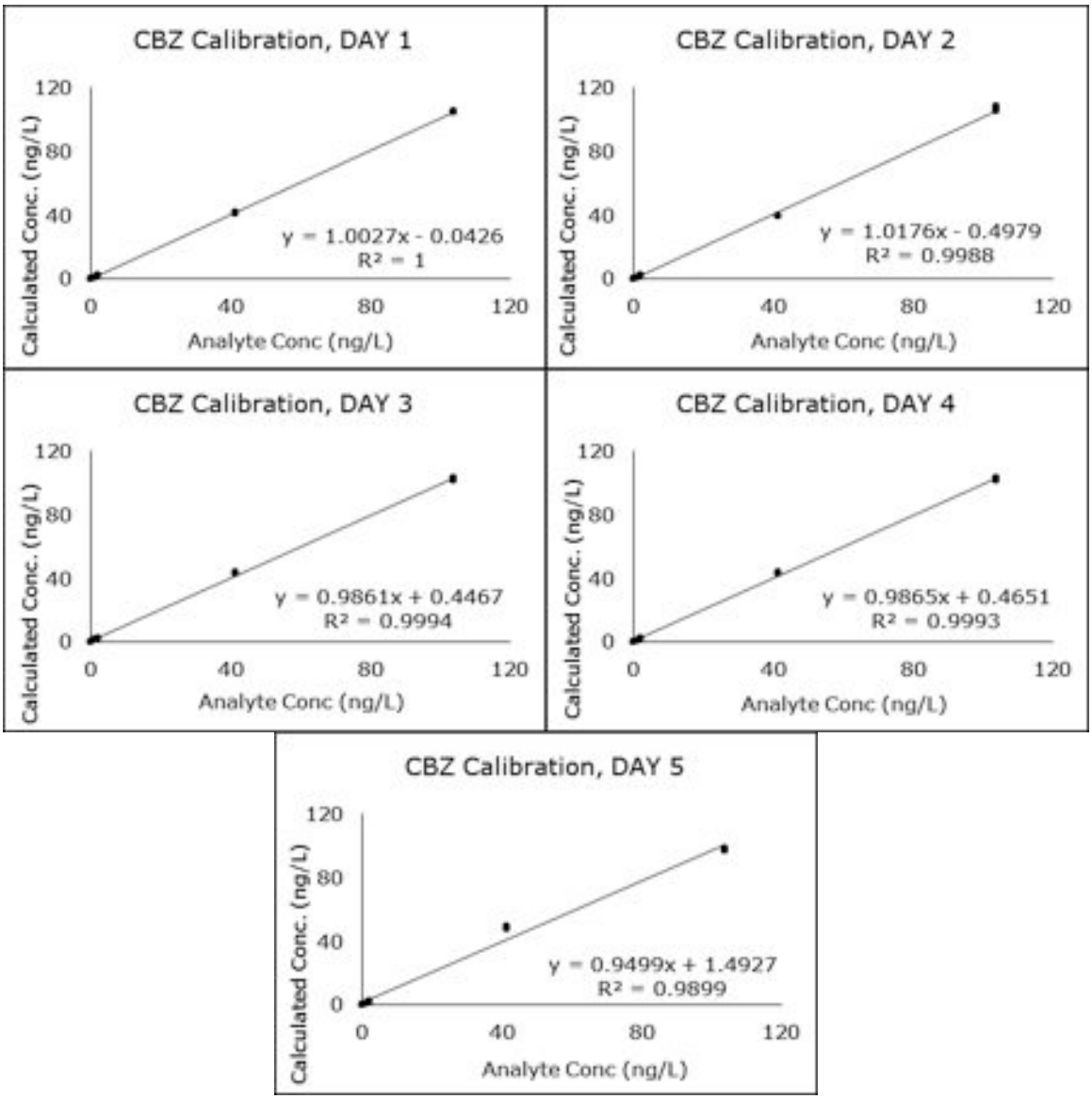


Figure 2 Residual plots of CBZ

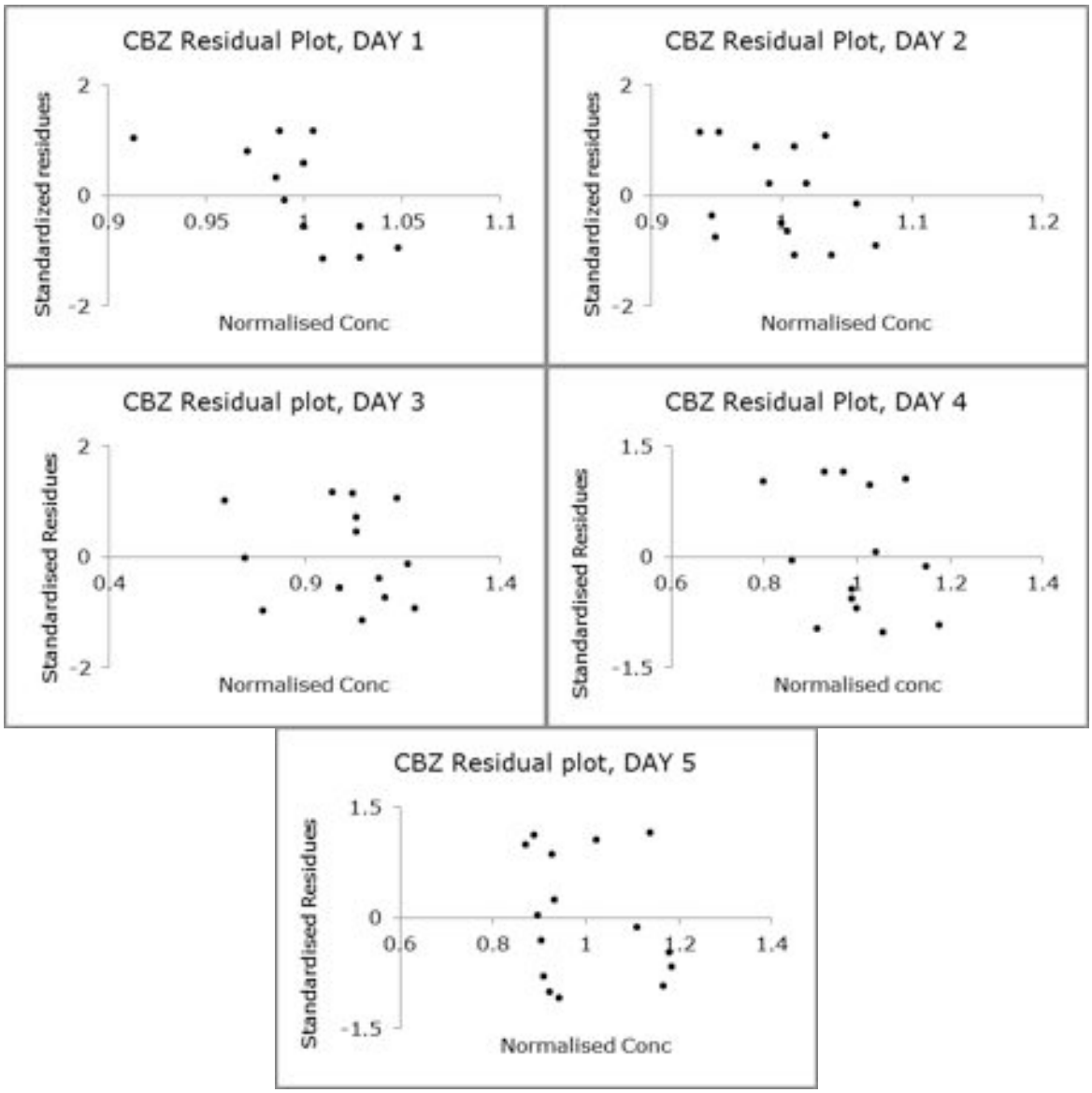


Figure 3 Calibration curves of CBZ-DiOH

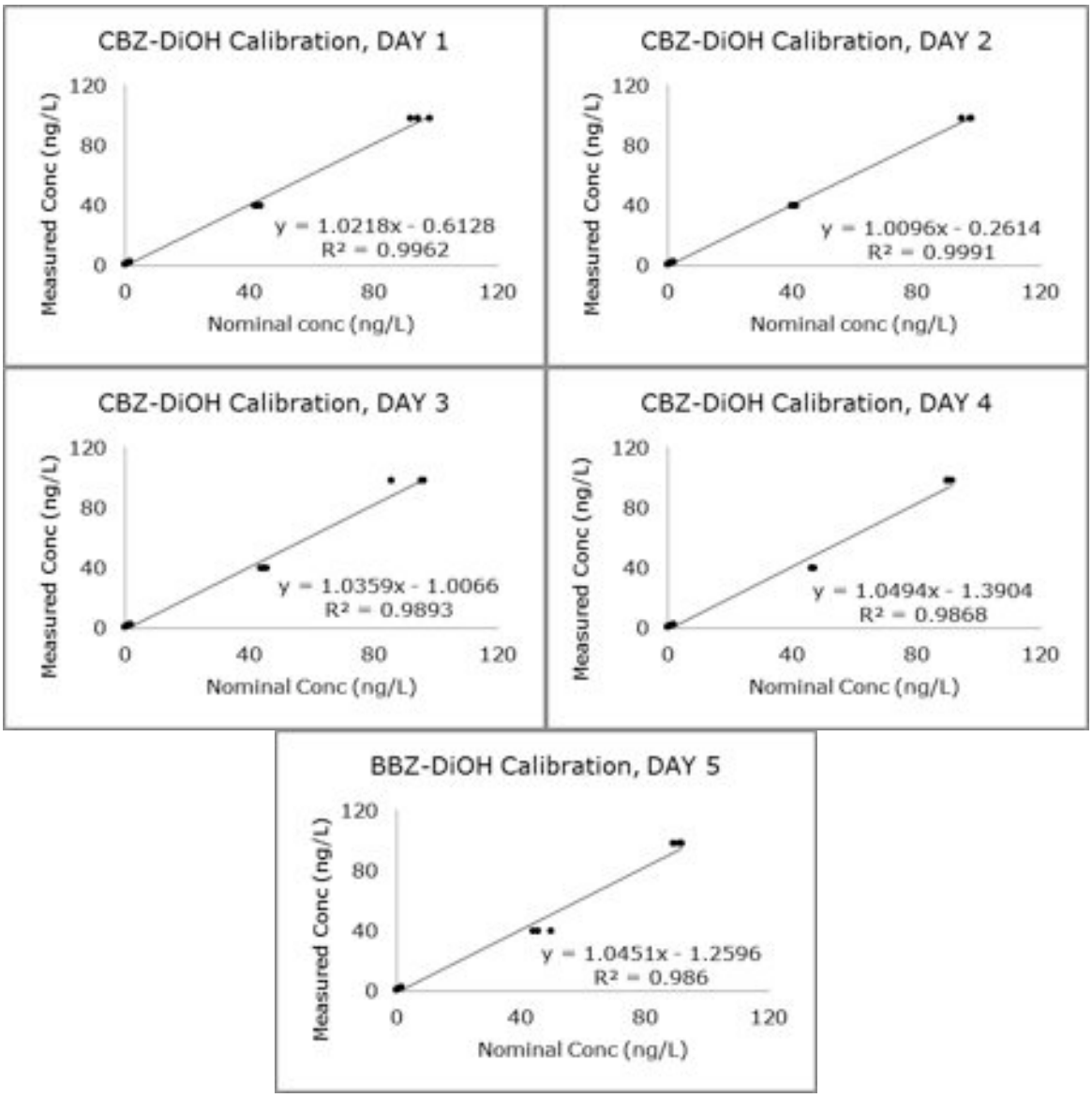


Figure 4 Residual plots of CBZ-DiOH

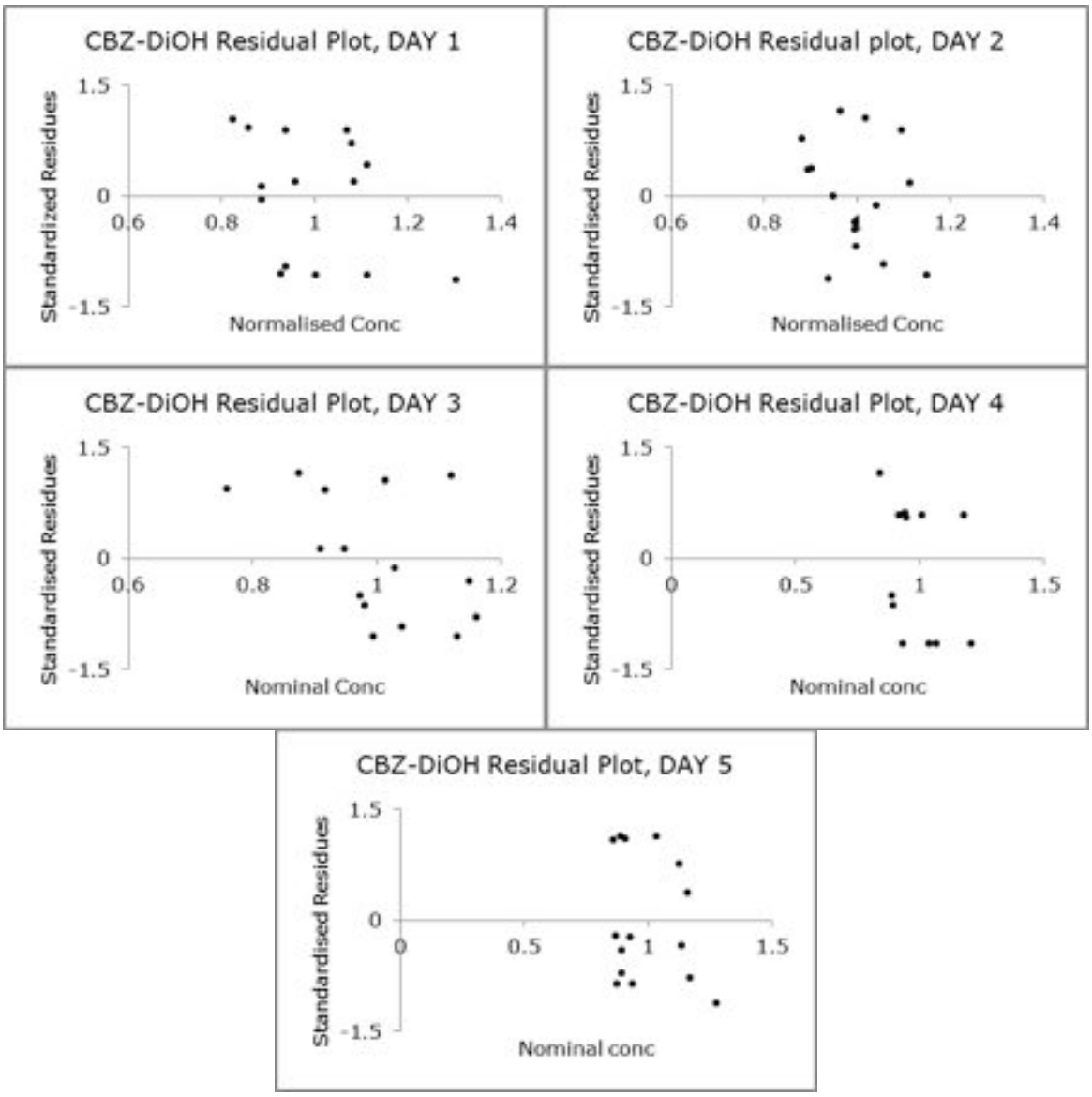


Figure 5 Calibration curves of SMZ

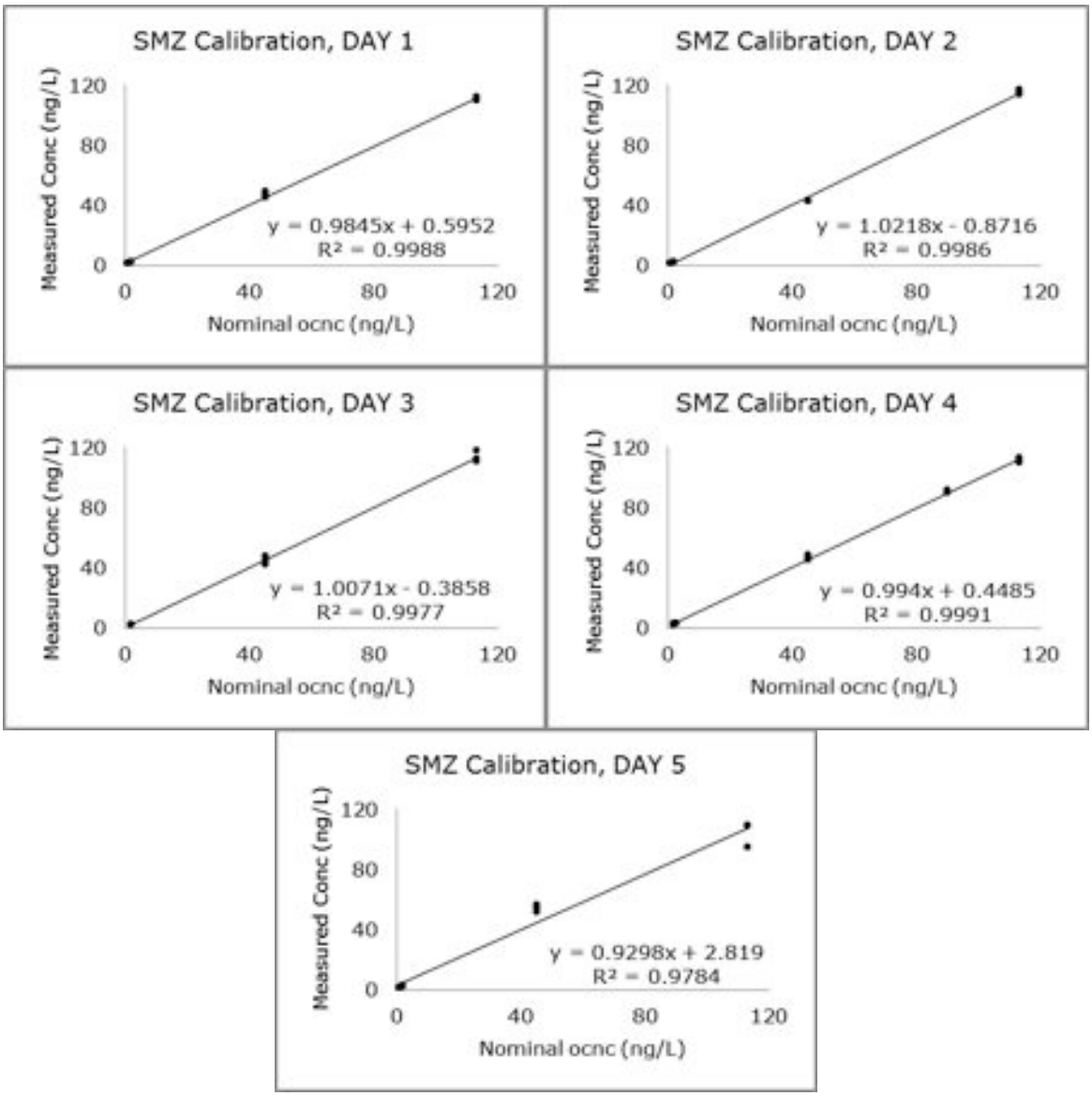


Figure 6 Residual plots of SMZ

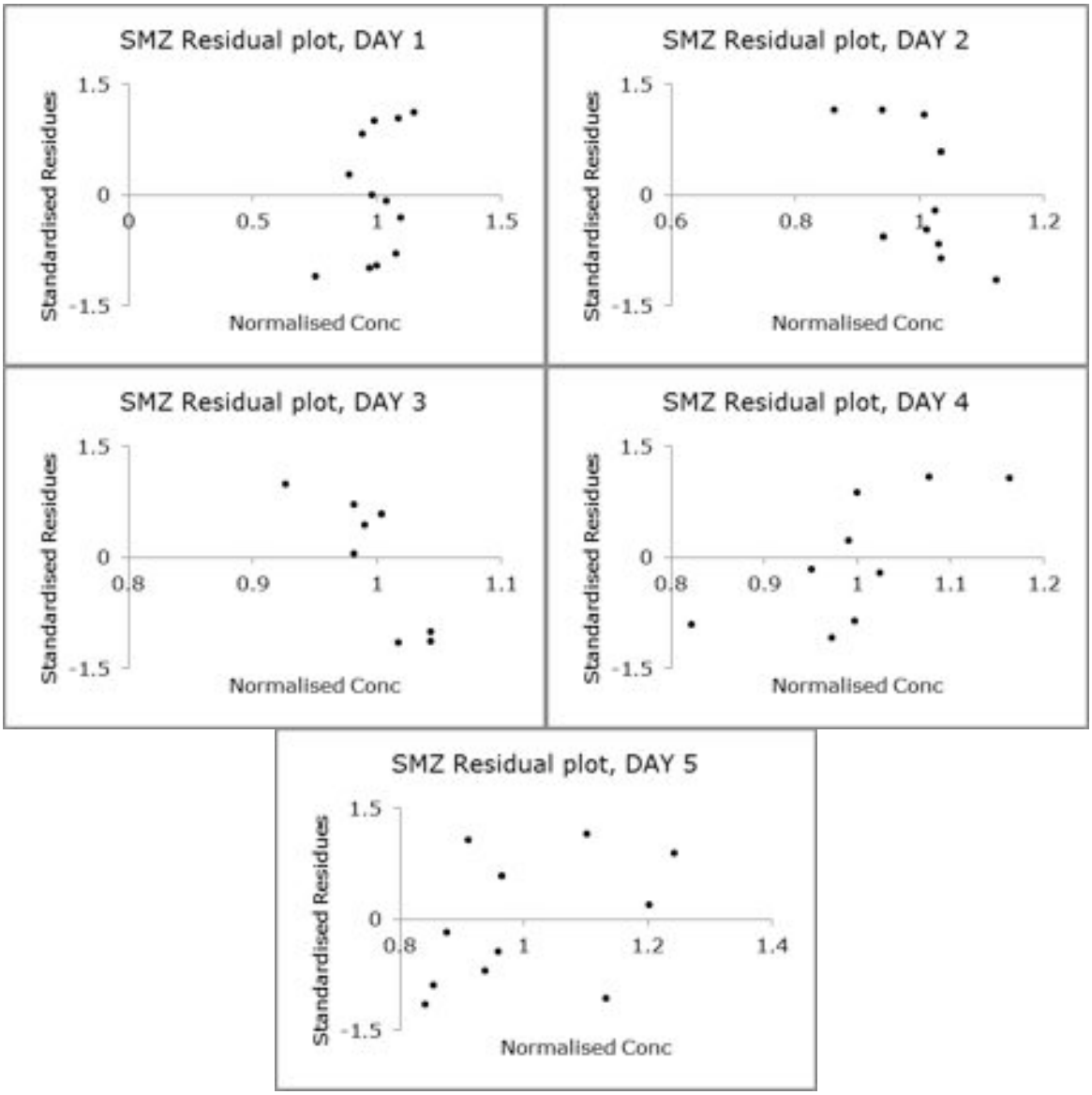


Figure 7 Calibration curves of TCPP

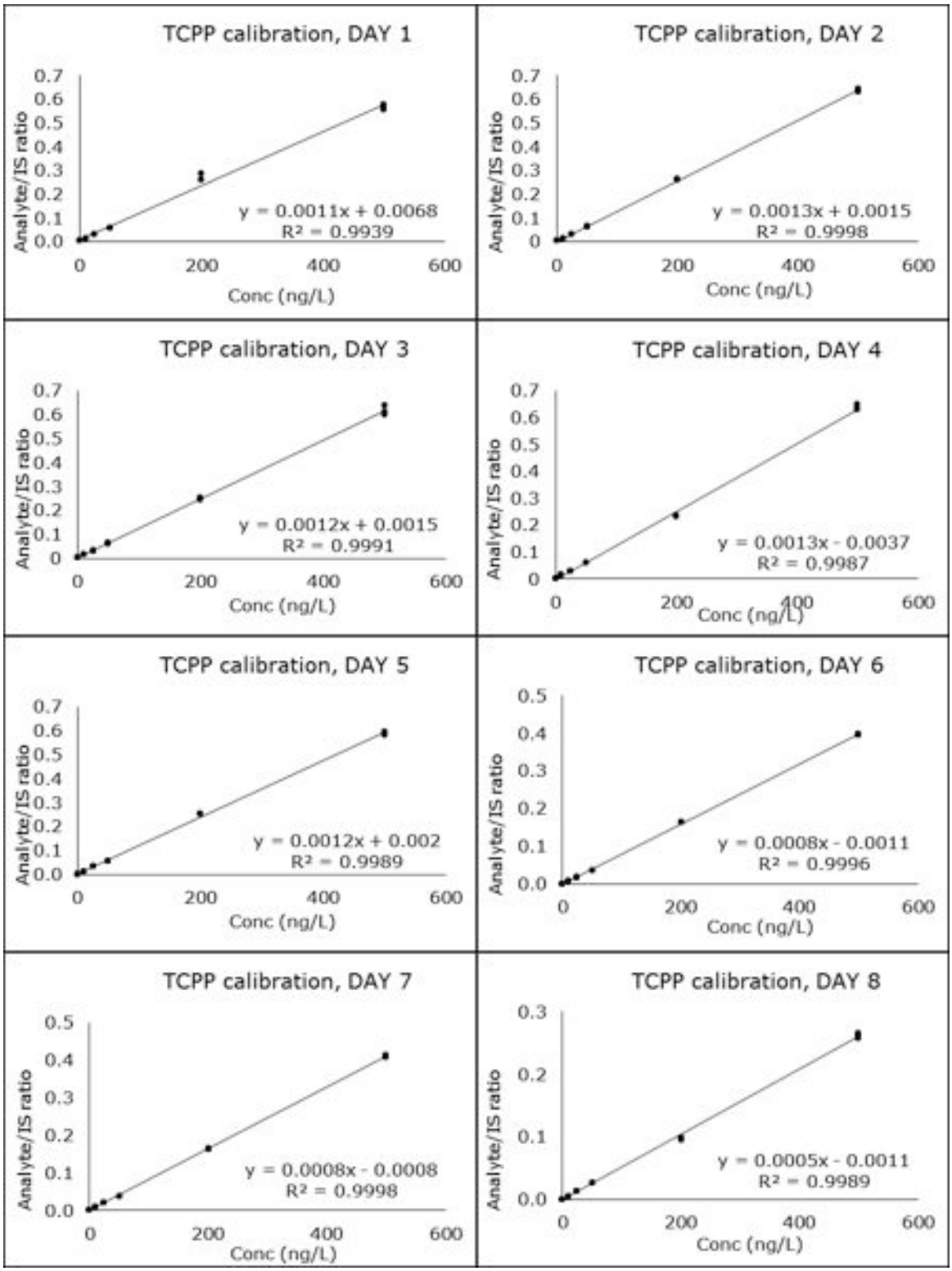


Figure 8 Residual plots of TCPP

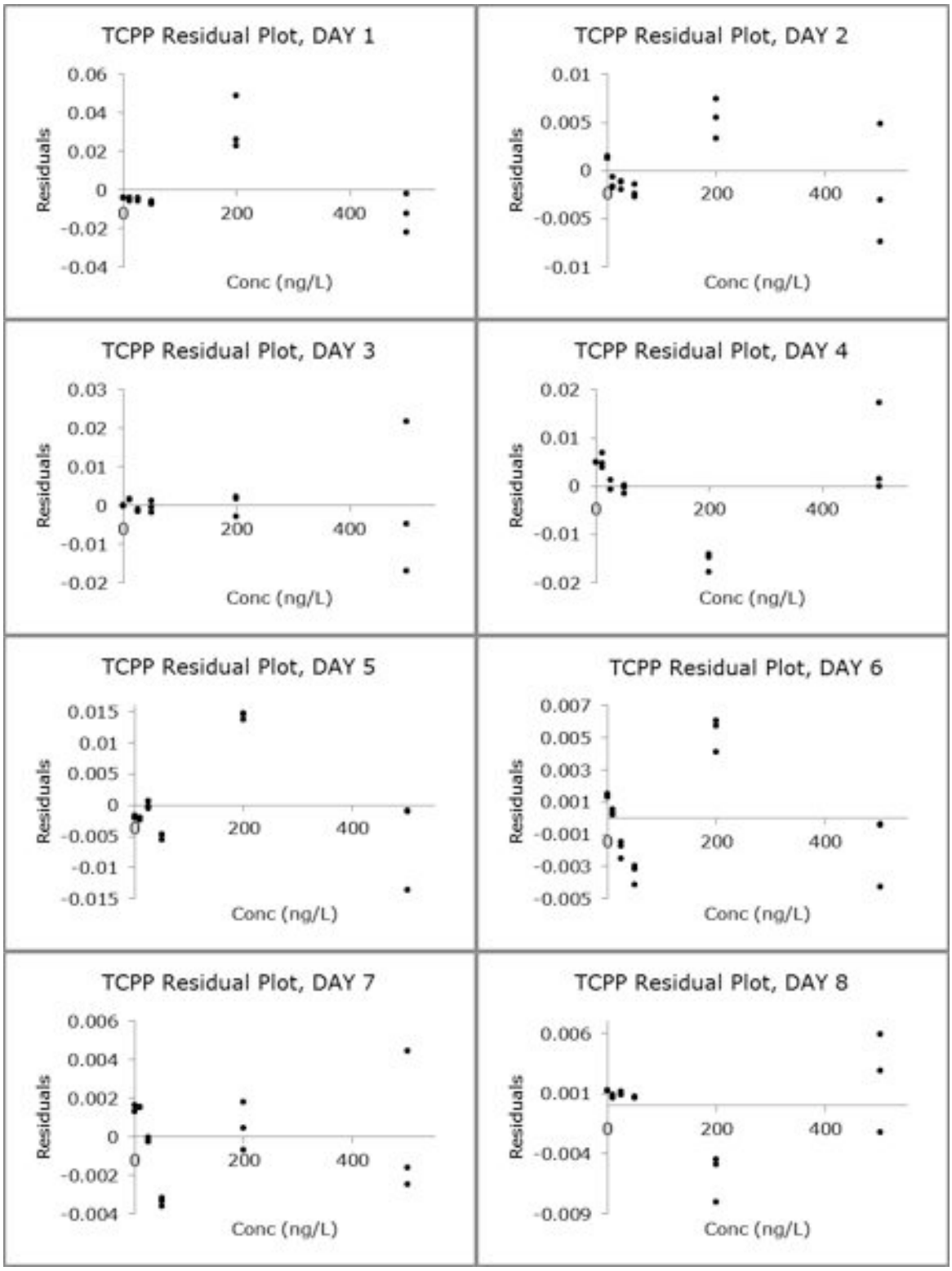


Figure 9 Calibration curves of PFPrA

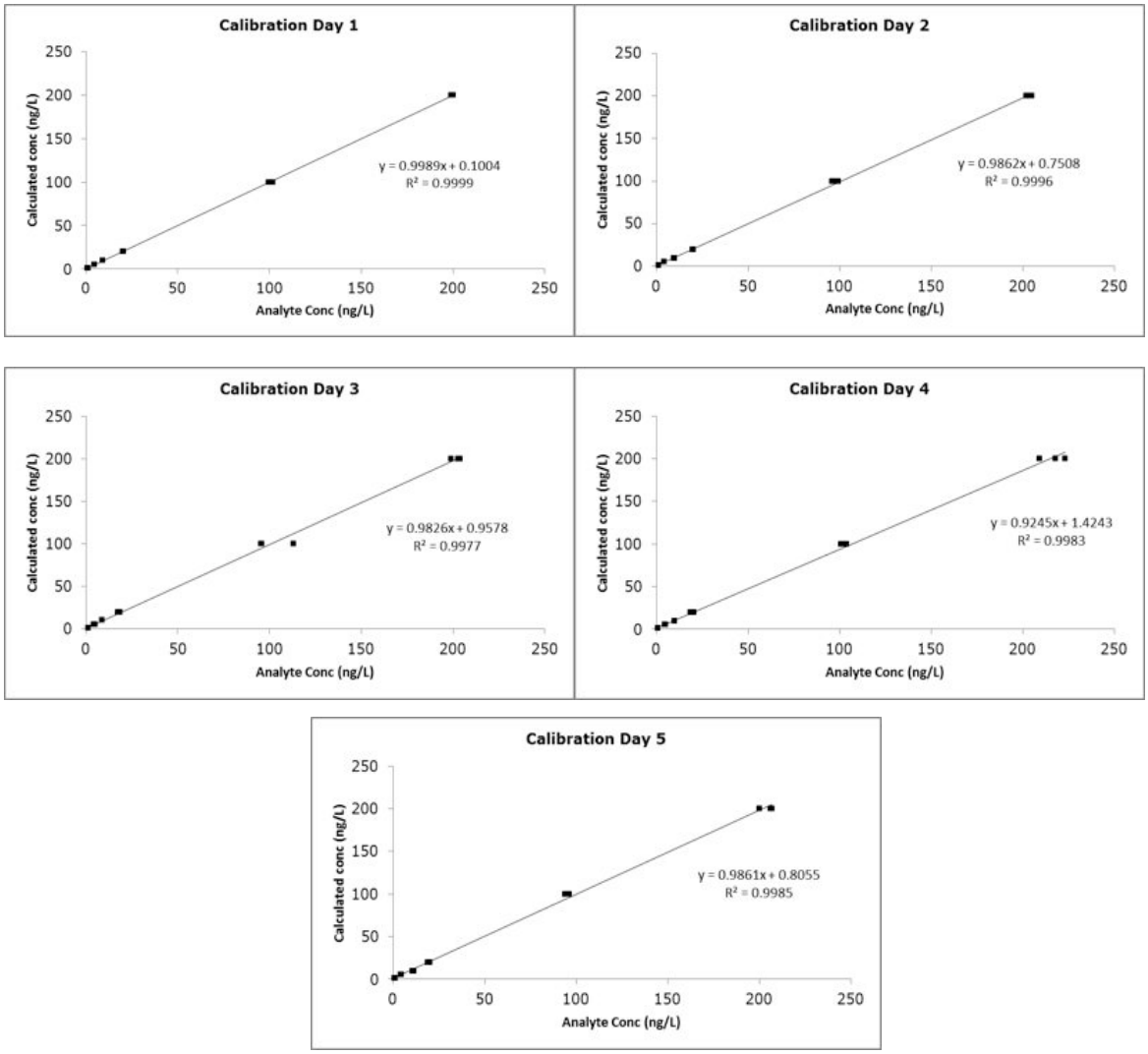


Figure 10 Residual plots of PFPrA

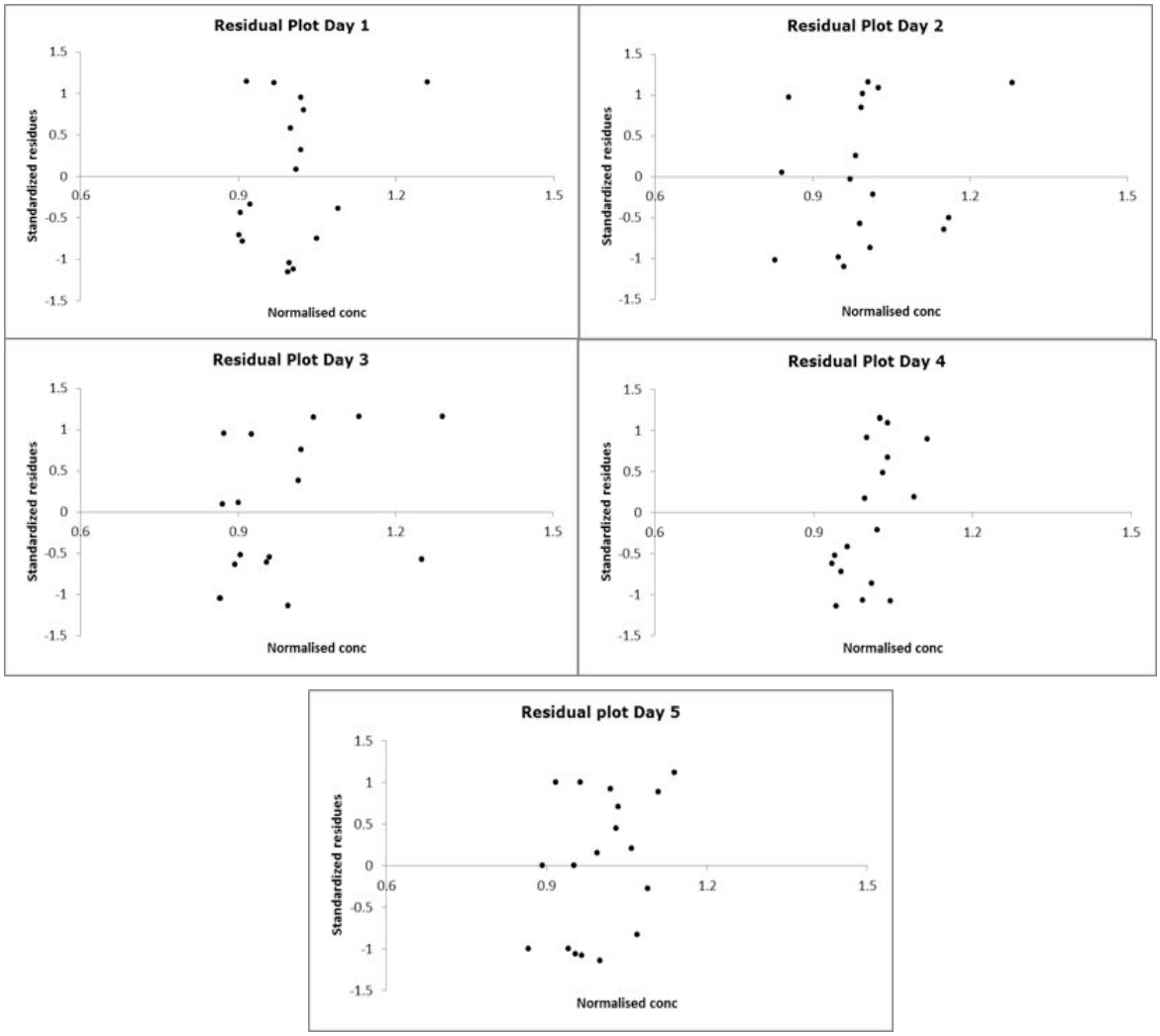
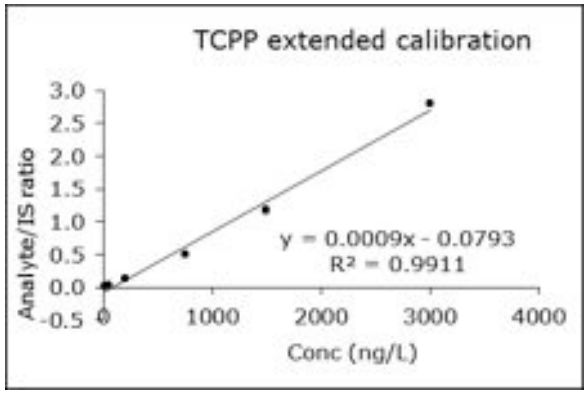
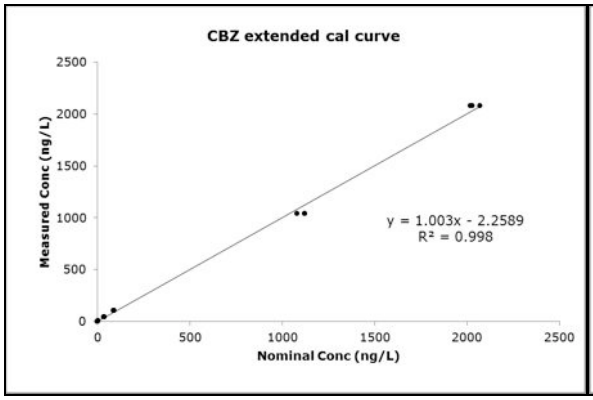
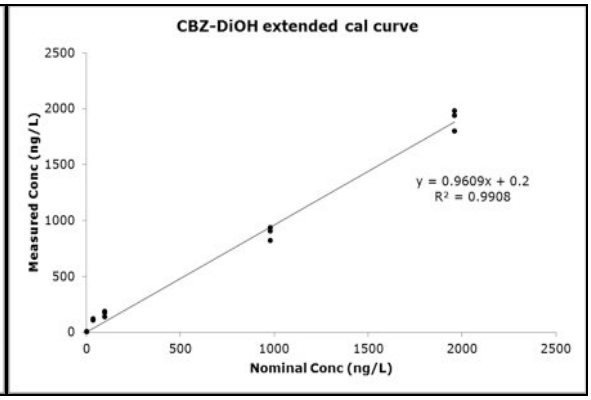


Figure 11 Extended calibration curves







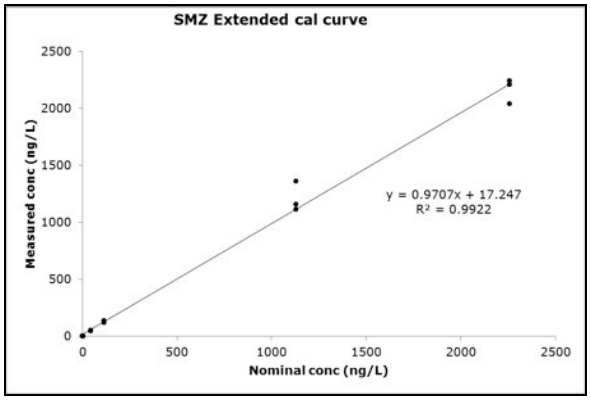


Figure 12 Results of CBZ, CBZ-DiOH and SMZ stability studies

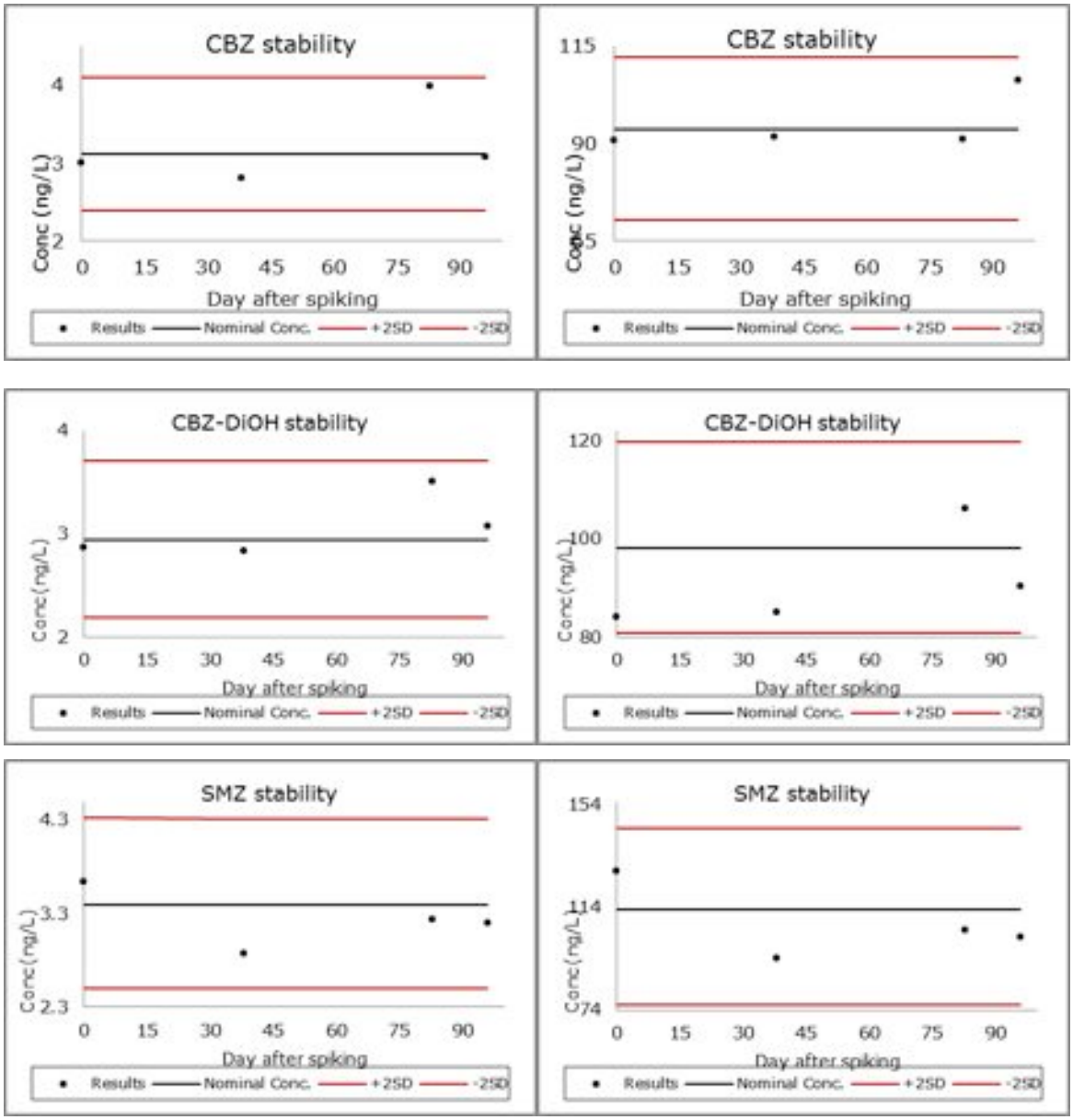
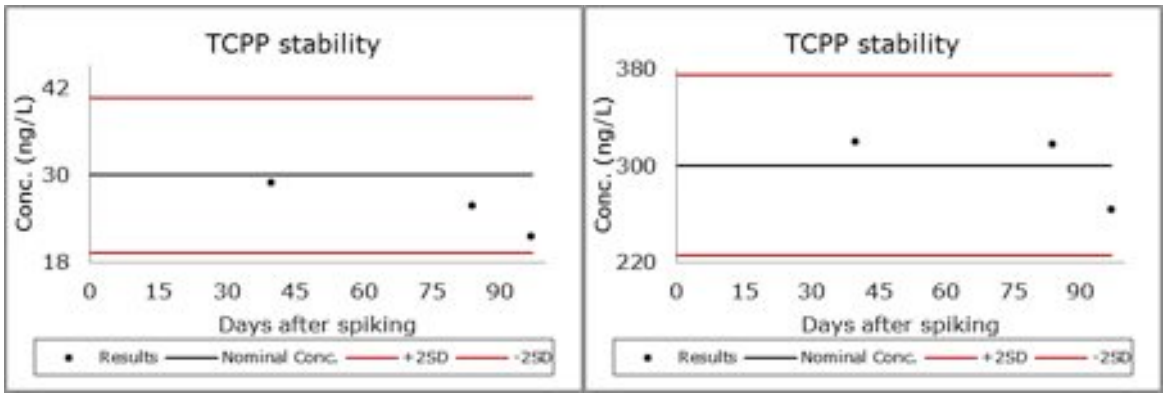


Figure 13 Results of TCPP stability study



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Abstract

Validation of an analytical method is a necessary step in controlling the quality of quantitative analysis. Method validation is an established process which is the provision of documentary evidence that a system fulfills its pre-defined specification or the process of providing that an analytical method is acceptable for its intended purpose.
The objectives of the present study were:

- to validate a SPE-LC-MS/MS method for the determination of carbamazepine (CBZ), 10,11-dihydro-10,11-dihydroxy-carbamazepine (CBZ-DiOH), sulfamethoxazole (SMZ) and pentafluoropropionic acid (PFPrA) in surface water samples;
- to validate a SPE-GC-MS method for the determination of tris (1-chloro-2-propyl) phosphate (TCPP) content in surface water samples.

Method validations were performed according to the ISO 17025 requirement and the BT/TF151 WI CSS 99026 document.
The calibration curves, working ranges, recoveries, detection and quantification limits, trueness as well as repeatability were determined.
The budget uncertainty was also estimated following a top-down approach based on in-house validation data.
The expanded combined relative uncertainty resulted as follows:

	Uncertainty (%)	
Analyte	**Low level spike**	**High level spike**
CBZ	35	39
CBZ-DiOH	34	27
SMZ	34	37
TCPP	32	24
PFPrA	26	32

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